## REMARKS/ARGUMENTS

### *Status of the Application*

In the Office Action, claims 31-34 were rejected and claims 25-30 and 35-46 were withdrawn from consideration because of a restriction requirement. In this Response, claim 31 was amended to form an independent claim. Further, the term "amino acid sequence" was amended to "isolated amino acid sequence," and the isolated amino acid sequence is now coded by nucleotides 112 to 462 of SEQ ID NO:11. As with claim 31, the term "amino acid sequence" was amended to "isolated amino acid sequence" in claims 32-34. In claim 32, references to withdrawn sequences have been removed. Claim 33 was amended to clarify that the fragment contains at least one contiguous amino acid from amino acids 37-117 of SEQ ID NO:32. In claims 33 and 34, the term "peptide" was amended to "isolated peptide."

Claims 47-48 were added (see page 4, lines 4-13, for support).

Thus, claims 31-34 and 46-47 are pending. No new matter was added.

### *Specification*

The specification has been corrected at page 20 to correct an obvious error made by Applicant in reciting the amino acid sequence corresponding to SEQ ID NO:32. Applicant notes that table 1 is correct in stating that ghrelin variant 2 has 117 amino acids. Applicant refers the Examiner to nucleotides 112 to 462 of SEQ ID NO:11, which encodes ghrelin variant 2. In translating the amino acid sequence of SEQ ID NO:11, Applicant mistakenly omitted the leucine residue found at amino acid position number 65 (between a histidine and a tryptophan). Nucleotides 304-306 of SEQ ID NO:11 as originally filed translates to the missing leucine residue.

Concurrently herewith, Applicant has submitted an updated sequence listing, a disk containing the updated sequence listing in computer readable format, and an accompanying sequence statement.

No new matter was added.

### *Claim Objections*

Applicant has amended claims 31-34 to remove references to non-elected inventions as described above. No new matter was added.

***Rejections Under 35 U.S.C. § 101***

Claim 31 was rejected under 35 U.S.C. § 101 as being directed to non-statutory subject matter, specifically the Examiner asserts that the term "an amino acid sequence" reads on a product of nature. Applicant has amended the term to read "an isolated amino acid sequence." Thus, Applicant respectfully submits that this rejection has been obviated.

Claims 31-34 were rejected under 35 U.S.C. § 101 as not being supported by either a specific and substantial asserted utility or a well-established utility. Applicant respectfully traverses these rejections.

Wild-type, human ghrelin is produced as a prepro-peptide of 117 amino acids. *See* Kojima M *et al.*, Nature **402**:656-60 (1999) (attached herewith). Cleavage of the prepro-peptide after amino acid 23 produces 94-amino acid pro-peptide (amino acids 24-117 of the full-length prepro-peptide). *Id.* Further cleavage of the pro-peptide produces a 28-amino acid peptide (amino acids 24-51 of the full-length prepro-peptide). *Id.* Post-translational acylation with octanoic acid at the second serine residue of the 28-amino acid peptide produces the fully processed ghrelin protein. *Id.*

Ghrelin has a well-established utility. Ghrelin is known by those of ordinary skill in the art to function, for example, as a stimulator of growth hormone secretion, as an orexigenic molecule, as an adipogenic molecule, as a protector of cardiac dysfunction, as a stimulator of gastric acid secretion, as a stimulator of gastric motility, as a modulator of circulating glucose release, as an enhancer of insulin resistance, as a stimulator of gluconeogenesis, and as an inducer of hunger sensations. *See, generally,* Ueno H *et al.*, Regul. Pept. **126**:11-19 (2005) (attached herewith). Ghrelin peptide fragments as small as five amino acids (Gly-Ser-Ser(*n*-octanoyl)-Phe-Leu) have been shown to be agonists of the growth hormone secretagogue receptor 1a, the receptor itself a mediator of growth hormone secretion. *See* Bednarek MA *et al.*, J. Med. Chem. **43**:4370-76 (2000) (attached herewith); *see also* Van Craenenbroeck *et al.*, Peptides **25**: 959-65 (2004) (attached herewith). Injection of a truncated porcine ghrelin variant into rats has been shown to increase body weight. *See* Xie QF *et al.*, Domest. Anim. Endocrinol. **27**:155-64 (2004) (attached herewith). Further, such fragments are commercially available. *See, e.g.,* Peptides International,

http://www.pepnet.com/ghrelin.html (last visited May 18, 2005) (attached herewith); Phoenix Pharmaceuticals, Inc., http://www.phoenixpeptide.com/cgi-bin/cartit/searchnew001d.cgi?dbsearch=searchnew.txt&field=6&catGroup=Peptides&string=Ghrelin (last visited May 18, 2005) (attached herewith).

***Rejections Under 35 U.S.C. § 112, 1st Paragraph***

Claims 31-34 were rejected under 35 U.S.C. § 112, 1st Paragraph, as not providing one skilled in the art with the knowledge to use the claimed inventions, because the claimed inventions allegedly were not supported by either a specific and substantial asserted utility or a well-established utility. Applicant respectfully submits that, because the utility rejection above has been traversed, the rejections under 35 U.S.C. § 112, 1st Paragraph, have also been traversed.

***Rejections Under 35 U.S.C. § 102(b)***

Claims 31-34 were rejected under 35 U.S.C. § 102(b) as being anticipated by Gualillo *et al.*, Endocrinology **142**:788-94 (2001). Applicant respectfully traverses these rejections.

Applicant has amended claim 31 to cover isolated amino acid sequences. Because Gualillo *et al.* do not teach an isolated amino acid sequence coded by nucleotides 112 to 462 of SEQ ID NO:11, and in fact only teach the wild type ghrelin sequence, Applicant respectfully submits that claim 31 is not anticipated by Gualillo *et al.*

Because claims 32-34 are dependent claims, which recite even further limitations to the claim that has already been traversed, Applicant relies upon the arguments presented above in rebuttal to the Examiner's assertion that claims 32-34 are anticipated by Gualillo *et al.*

Claims 31-34 were also rejected under 35 U.S.C. § 102(b) as being anticipated by Sheppard *et al.* (U.S. Patent No. 6,291,653). Applicant respectfully traverses these rejections.

Claim 31 has been amended to cover an isolated amino acid sequence coded by nucleotides 112 to 462 of SEQ ID NO:11. Because Sheppard *et al.* do not disclose an amino acid sequence encoded by nucleotides 112 to 462 of SEQ

ID NO:11, Applicant respectfully submits that Sheppard *et al.* does not anticipate claim 31.

Because claims 32-34 are dependent claims, which recite even further limitations to the claim that has already been traversed, Applicant relies upon the arguments presented above in rebuttal to the Examiner's assertion that claims 32-34 are anticipated by Sheppard *et al.*

***Summary***

In view of the foregoing amendments and remarks, Applicant submits that this application is in condition for allowance. In order to expedite disposition of this case, the Examiner is invited to contact Applicant's representative at the telephone number below to resolve any remaining issues. Should there be a fee due which is not accounted for, please charge such fee to Deposit Account No. 501447 (Potter Anderson & Corroon LLP).

Respectfully Submitted,

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# Ala-scan of ghrelin (1–14): interaction with the recombinant human ghrelin receptor

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**Abstract**

Ghrelin, a 28 residues acylated peptide, is the natural ligand of the growth-hormone secretagogue receptor (GHS-R), which also interacts with small synthetic peptides. We investigated the importance of each of the first 14 N-terminal residues by Ala replacement (Ala-scan) and also of the N-terminal positive charge, on the recombinant GHS-R expressed in HEK293 or CHO cells by binding, IP and $Ca^{2+}$ assays. Nearly all of the replacements had no significant effect on the ligand binding or $IP_3/Ca^{2+}$ stimulation. Exceptions were the modification of the N-terminal residue to $[A_1]$- or $N^{\alpha}$-acetyl-ghrelin (1–14), confirming the requirement for the positive charge at the amino-terminus. Mutation of $[F_4]$- to $[A_4]$- or $[Y_4]$-ghrelin (1–14), were detrimental suggesting direct interaction with the GHS-R. $[A_8]$ and $[Y_8]$ were more potent than ghrelin (1–14), implying that the naturally occurring $Glu^8$ residue may not be the optimal.


*Keywords:* Ghrelin; GHS-receptor; Ala-scan

## 1. Introduction

Ghrelin is a 28 amino acid peptide [13] generated by the processing of a precursor expressed mainly in the stomach, but also in the hypothalamus arcuate nucleus [13,22]. Ghrelin as a 27 amino acids peptide can be found as well, resulting from the removal of the carboxyl-terminal Arg during precursor cleavage [11] or through alternative splicing removing $Glu^{14}$ residue [10]. Ghrelin's main structural originality is the occurrence of an acyl group esterifying the hydroxyl function of the Ser residue in position 3. A bulky hydrophobic group (e.g. octanoylation, decanoylation, or dodecanoylation) at position 3 is absolutely required for biological activity [3,11]. Ghrelin is the endogenous ligand of an orphan receptor of the GPCR I group (rhodopsin group) identified originally through its interaction with small synthetic molecules called growth-hormone secretagogue (GHS) [17]. Its activation stimulates growth-hormone release from the pituitary gland independently of growth-hormone releasing peptide, the latter acting through interaction with the GHRH receptor, a member of the GPCR II group (secretin receptor family) [17,19]. Much is known of the interaction of small non-ghrelin ligands with the receptor, derived from endorphin like molecules (e.g. GHRP-6, hexarelin), that have been used to identify the receptor. However, in vivo studies showed that bioavailability of the GHS peptides are low [1], therefore non-peptide compounds such as MK-0677 and L-692,429 were developed [20]. Nevertheless, limited data is available on the structure–function relationships of the natural ligand ghrelin and analogs thereof [8]. Though recent studies have investigated truncated ghrelin peptides [3,14,15,23] and have revealed the importance of the first 14 N-terminal residues, no systematic study of the critical residues required for receptor binding and activation has been carried out. Consequently, it seemed propitious to determine the exact contacts between ghrelin and its receptor. The purpose of the present work was therefore to systematically test the importance of the first 14 amino acid residues and of the amino-terminal positive charge of ghrelin on ghrelin receptor recognition, inositol phosphates synthesis and calcium mobilization in CHO cells stably transfected and in HEK293 cells transiently transfected with the recombinant human ghrelin receptor. Ultimately, this may favor design of useful non-peptide compound drugs specific only for the ghrelin receptor.

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## 2. Materials and methods

### 2.1. Synthesis of ghrelin (1–14) analogs

The peptides were synthesized by the solid phase method using the Fmoc (fluoro-methoxy carbonyl) strategy as previously described [9] on a Symphony PTI Multiplex synthesizer. Fmoc is a base-labile group used to protect the α-amino group. Acid-labile side-chain protecting groups such as *tert*-butyl for Ser and Tyr, trityl for His, Glu and $Ser^3$ were used to avoid secondary reactions on the reactive functional groups. The SPPS technique provides stepwise assembly of single residue addition. The first step is the attachment of the C-terminal amino acid to the resin linker. Next, the Fmoc of the attached amino acid is removed by treatment with 20% v/v piperidine in DMF (dimethylformamide). An excess of the second amino acid is introduced with the carboxy-group of this amino acid being activated for amide bond formation through generation of an activated ester using a mixture of NMM/HBTU (NMM: *N*-methyl morpholine, HBTU: *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyl-uronium PF6) in DMF/NMP (*N*-methyl pyrolidone). The coupling reaction can take as much as 3 h to be completed. A solution of 20% of acetic anhydride is then used to cap unreacted amino acids prior to addition of the third amino acid residue. This process is repeated until the desired peptide is assembled.

### 2.2. Octanoylation of the serine in position 3 [Octa]$S_3$

Octanoylation was performed as described [14] with slight modifications. We used trityl for protection of the alcohol function of $Ser^3$. The fully protected peptidyl resin was manually treated with a solution of 1% TFA in dichloromethane (DCM) to free the alcohol. The peptidyl resin was filtered and washed with fresh DCM, before octanoylation itself. The reaction was performed at room temperature for 3 h using a solution of a sixfold excess of octanoic acid and WSC-HCl in DCM in the presence of a fivefold excess of DMAP. The *O*-acylated ghrelin was washed with fresh DCM and NMP, before removal of the Fmoc group of the N-terminal amino acid, using piperidine solution. The peptidyl resin was cleaved and all protecting groups were removed by a treatment with a cocktail of 80% (v/v) TFA, 5% (v/v) $H_2O$, 5% (v/v) thioanisole, 5% (v/v) triisopropylsilane, 5% (v/v) phenol and 2.5% (v/v) ethane dithiol. After 3 h, the resin was filtered off and the peptide was precipitated in cold ether. The residue was washed several times with fresh ether and purified by RP-HPLC using semi-preparative and analytical C18 columns. Peptide purity was assessed by capillary electrophoresis on a PACE MD Beckman instrument, and peptide mass spectrometry on a Micromass Platform II. Amino acids and polystyrene resin (MBHA) were purchased from Novabiochem (Switzerland). All reagents and solvents were of analytical grade. GHRP-6 was purchased from Bachem (Switzerland).

### 2.3. Calcium assay

Calcium increase was measured by a functional assay based on the luminescence of mitochondrial aequorin/coelenterazine in response to calcium increase as previously described [3,6,7,21]. A stable CHO cell line expressing the aequorin protein (courtesy of Euroscreen, Brussels, Belgium), and stably transfected with the ghrelin receptor tagged at its carboxyl-terminus by a green fluorescent protein (EGFP), contained in the eukaryotic expression vector pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA, USA) was used. The tagged GHS-R receptor was originally constructed to follow receptor expression in receptor mutagenesis and desensitization experiments. In the experiments herein, EGFP was used only to monitor transient expression qualitatively. Fluorescence quantification was not judged necessary in these experimental settings, likewise for the stable CHO clone. The GHS-R/EGFP tagged receptor was extensively characterized beforehand (data not shown) by calcium increase and inositol phosphates accumulation in the presence of ghrelin (1–28), desoctanoylated ghrelin (1–28), ghrelin (1–23), ghrelin (1–14) and desoctanoylated ghrelin (1–14) and compared to cell lines expressing the wild-type receptor (i.e. untagged). The $EC_{50}$ values were not different between both cells types. Briefly, cells were collected from plates in 1× PBS pH 7.4 containing 5 mM EDTA, pelleted and resuspended at $5 \times 10^6$ cells/ml in DMEM-F12 medium (containing 1 mM $Ca^{2+}$) supplemented with 0.5% BSA; then incubated with 5 μM coelenterazine H (Molecular Probes) for 3 h at room temperature under light agitation. Cells were then diluted 10-fold and incubated for one more hour. Fifty microliters of cell suspension were added to agonists diluted in a volume of 50 μl DMEM-F12. Calcium increase was evaluated by measuring the luminescent signal for 20 s and integration of area under the curve resulting from the activation of the aequorin/coelenterazine complex, using a microlumat luminometer (Perkin-Elmer). The data were normalized for basal (0%, background removal) and maximal luminescence (100%) corresponding to the signal measured following exposure to 50 μM Digitonin. Calcium increase following 10 μM ATP stimulation of a constitutively expressed receptor was also measured, in separate replicate wells, to verify cell integrity.

### 2.4. Inositol phosphates assay

The same CHO cell line as used for the calcium assay was used for the inositol phosphates assay. The CHO cells were incubated in a 24-wells plate in the presence of [$^3$H]myoinositol 48–72 h at 37 °C. The labeled cells were then washed with 0.250 ml DME medium and incubated for 30 min with 10 mM LiCl at room temperature. After this preincubation, cells were incubated in the presence of 30 μl agonist for 20 min at 37 °C. The reaction was terminated by removing the medium and then adding 0.500 ml of chilled

methanol (−20 °C). The wells were scrapped and rinsed with an equal volume of methanol. To the pooled methanol sample, 2 ml of $CHCl_3$ and 1 ml $H_2O$ were added. The mixture was then vortexed to obtain two phases. The aqueous phase was collected and the inositol phosphates were eluted by anion exchange chromatography on Dowex AG 1-X8 (200–400 mesh formate form) as previously described [4].

### 2.5. Tracer and binding studies

The tracer we developed and used was iodinated [$Y_{24}$]-ghrelin (1–23) ([$^{125}$I][$Y_{24}$]-ghrelin (1–23)). The unlabeled peptide had an $IC_{50}$ of ±15 nM. [$Y_{24}$]-ghrelin (1–23) was successfully iodinated by the iodogen method as described previously for other peptides [9]. The radioactive peptide was separated from free iodine by adsorption on a Sep-pak and elution with 50% acetonitrile in 0.5% TFA.

Binding obtained on stable CHO cell lines was considered to low for accurate competition curves. On the other hand, useful and reproducible data were obtained from transiently transfected HEK293 cells. Therefore, competition curves were performed for all the analogs on these cell membranes. HEK293 cells as well as CHO cells non-transfected and transfected by an irrelevant cDNA were tested for tracer binding and neither cell type bound the tracer (data not shown).

For the binding studies HEK293 cells were detached with PBS/EDTA solution 72 h after transfection, pelleted and lysed in 1 mM $NaHCO_3$ solution followed by immediate freezing in liquid nitrogen. After thawing, the lysate was centrifuged for 15 min at 25,000 × *g*. The pellet was resuspended in the bicarbonate solution and used immediately as a crude membrane fraction. Binding studies were performed at 25 °C for 30 min, in a total volume per assay of 0.120 ml consisting in a 20 mM Tris/maleate buffer, 2 mM $MgCl_2$, 0.1 mg/ml bacitracin, 1% bovine serum albumin, 25,000 cpm of tracer (final concentration of 0.03 nM) (pH 7.6), increasing concentrations of unlabeled peptide competitors and 3–30 μg membrane protein.

Bound and free radioactivity were separated by filtration through glass-fiber GF/C filters presoaked for 24 h in 0.01% polyethyleneimine and rinsed three times with ice-cold 20 mM sodium phosphate buffer pH 7.4 containing 1% bovine serum albumin. The non-specific binding was defined as the residual binding in presence of 1 μM unlabeled ghrelin (1–14) and never exceeding 10% of the total radioactivity bound.

### 2.6. Analysis of results

Binding curves and dose–effect curves were analyzed by non-linear regression. $pEC_{50}$ and $pIC_{50}$ values are presented as the mean ± S.E.M. of 3–23 determinations. Correla-

Table 1
Summary of effect on $Ca^{2+}$ induced aequorin luminescence and IP accumulation assay in CHO, and specific [$^{125}$I][$Y_{24}$]-ghrelin (1–23) binding (mean ± S.E.M.; $n$ = 3–23)

| Peptides | IP | | $Ca^{2+}$ | | Binding |
|---|---|---|---|---|---|
| | $pEC_{50}$ | $E_{max}$ (%)[a] | $pEC_{50}$ | $E_{max}$ (%)[a] | $pIC_{50}$ |
| GHRP-6 | 6.42 ± 0.30 | 93 ± 6.0 | 8.70 ± 0.18** | 88 ± 11 | 6.72 ± 0.02 |
| Ghrelin (1–28) | 7.61 ± 0.21** | 79 ± 6 | 8.45 ± 0.30** | 77 ± 4 | 7.98 ± 0.04** |
| Ghrelin (1–23) | 7.93 ± 0.06** | 86 ± 8 | 8.36 ± 0.08** | 93 ± 17 | 7.44 ± 0.08** |
| Ghrelin (1–14) | 6.54 ± 0.11 | 100 | 7.68 ± 0.09 | 100 | 6.66 ± 0.05 |
| Desoctanoylated ghrelin (1–14) | – | – | – | – | – |
| $N^{\alpha}$-Acetyl-ghrelin (1–14) | – | 16 ± 11** | 6.39 ± 0.09** | 40 ± 6** | 5.99 ± 0.11** |
| [$A_1$]-ghrelin (1–14) | 6.68 ± 0.09 | 92 ± 6 | 8.28 ± 0.11** | 92 ± 1 | 7.00 ± 0.03 |
| [$A_2$]-ghrelin (1–14) | 6.76 ± 0.06 | 93 ± 16 | 8.30 ± 0.08** | 96 ± 1 | 7.00 ± 0.08 |
| [$A_4$]-ghrelin (1–14) | – | – | 5.60 ± 0.09** | 89 ± 13 | – |
| [$A_5$]-ghrelin (1–14) | 6.58 ± 0.29 | 74 ± 5 | 7.94 ± 0.18 | 86 ± 1 | 6.40 ± 0.20 |
| [$A_6$]-ghrelin (1–14) | 6.42 ± 0.12 | 98 ± 11 | 8.18 ± 0.12 | 86 ± 1 | 6.71 ± 0.19 |
| [$A_7$]-ghrelin (1–14) | 6.28 ± 0.12 | 98 ± 5 | 8.05 ± 0.08 | 89 ± 6 | 6.51 ± 0.11 |
| [$A_8$]-ghrelin (1–14) | 7.08 ± 0.08 | 108 ± 16 | 8.40 ± 0.12** | 88 ± 2 | 7.46 ± 0.18** |
| [$A_9$]-ghrelin (1–14) | 6.56 ± 0.17 | 81 ± 9 | 7.75 ± 0.04 | 83 ± 5 | 6.75 ± 0.20 |
| [$A_{10}$]-ghrelin (1–14) | 6.80 ± 0.13 | 103 ± 18 | 8.05 ± 0.07 | 84 ± 3 | 6.66 ± 0.09 |
| [$A_{11}$]-ghrelin (1–14) | 6.36 ± 0.03 | 96 ± 11 | 7.64 ± 0.16 | 87 ± 2 | 6.60 ± 0.14 |
| [$A_{12}$]-ghrelin (1–14) | 6.84 ± 0.04 | 89 ± 11 | 8.07 ± 0.08 | 93 ± 7 | 6.64 ± 0.20 |
| [$A_{13}$]-ghrelin (1–14) | 6.95 ± 0.09 | 97 ± 11 | 8.00 ± 0.15 | 87 ± 11 | 6.66 ± 0.18 |
| [$A_{14}$]-ghrelin (1–14) | 6.97 ± 0.04 | 102 ± 14 | 8.13 ± 0.16 | 83 ± 4 | 6.47 ± 0.25 |
| [$Y_4$]-ghrelin (1–14) | 5.35 ± 0.01** | 89 ± 10 | 6.94 ± 0.07** | 75 ± 2.1 | 6.25 ± 0.01 |
| [$Y_8$]-ghrelin (1–14) | 8.38 ± 0.27** | 95 ± 5 | 8.78 ± 0.18** | 89 ± 8 | 7.66 ± 0.04** |
| [$Y_{14}$]-ghrelin (1–13) | 6.62 ± 0.32 | 85 ± 10 | 8.52 ± 0.15** | 80 ± 6 | 6.76 ± 0.02 |
| [$Y_{24}$]-ghrelin (1–23) | 8.12 ± 0.32** | 77 ± 16 | 8.59 ± 0.14** | 79 ± 1.4 | 7.77 ± 0.03** |

Endash indicates that the analogs tested were inactive or that their $pEC_{50}$ or $pIC_{50}$ values could not be validly determined.

[a] $E_{max}$ is expressed as % of the values obtained at 1000 nM ghrelin (1–14).

** Different from ghrelin (1–14), $P < 0.01$ (one-way ANOVA and Dunnett's post test).

tions between calcium or inositol phosphate assay $pEC_{50}$ values against binding $pIC_{50}$ values were carried out using Pearson's test. Statistical comparisons between $pEC_{50}$ or $pIC_{50}$ values were obtained by one-way ANOVA followed by Dunnette's test. All the graphics and statistical values reported were obtained using GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego, CA, USA.

## 3. Results

### 3.1. Ghrelin reference peptides

The $pEC_{50}$ values for calcium increase and inositol phosphates accumulation together with the $pIC_{50}$ values for binding in the presence of ghrelin (1–28), desoctanoylated ghrelin (1–28), ghrelin (1–23), ghrelin (1–14) and desoctanoylated ghrelin (1–14) are presented in Table 1. The complete dose–effect curves and binding competition curves are presented in Fig. 1. Ghrelin (1–28) was the most potent peptide when tested by the most sensitive parameter, i.e. $[Ca^{2+}]_i$ increase. Ghrelin (1–14) was sevenfold less potent than the complete molecule, but the three peptides were similarly efficient. On inositol phosphates accumulation, ghrelin (1–14) was 14-fold less potent than the two longer peptides that were equipotent. Desoctanoylated ghrelin (1–28) was 300- to 1000-fold less potent than the octanoylated form (data not shown) and for the shorter forms ((1–23) and (1–14)) the potencies were so low that they could not be estimated (Fig. 1). As the contribution of the carboxy-terminus to the peptide potency was limited and octanoylation being essential, we therefore decided to perform an "Ala-scan" of the 1–14 form with the octanoylated $Ser^3$ residue maintained.

### 3.2. Ala-scan of ghrelin (1–14): functional data

We considered that at least two of the three assays (i.e. intracellular calcium mobilization, inositol phosphate increase and binding) had to be statistically significant, with respect to the ghrelin (1–14) reference peptide to be considered biologically relevant. Accordingly, intracellular calcium and IP $pEC_{50}$ values, as well as binding $pIC_{50}$ were not significantly different from ghrelin (1–14) reference peptide for nearly all Ala substitutions of residues 1–14, with $[A_1]$-ghrelin $[A_2]$-ghrelin being four- to fivefold more potent in the calcium assay only. In contrast, in all assays, $Ala^4$ was totally detrimental and $Ala^8$ improved four- to sixfold the peptide's affinity and potency. In the calcium assay, $[A_4]$-ghrelin (1–14) was 100-fold less potent than ghrelin (1–14), but the extrapolated dose–effect curves suggested that the maximal response was maintained (Fig. 2). Similar conclusions could be drawn from

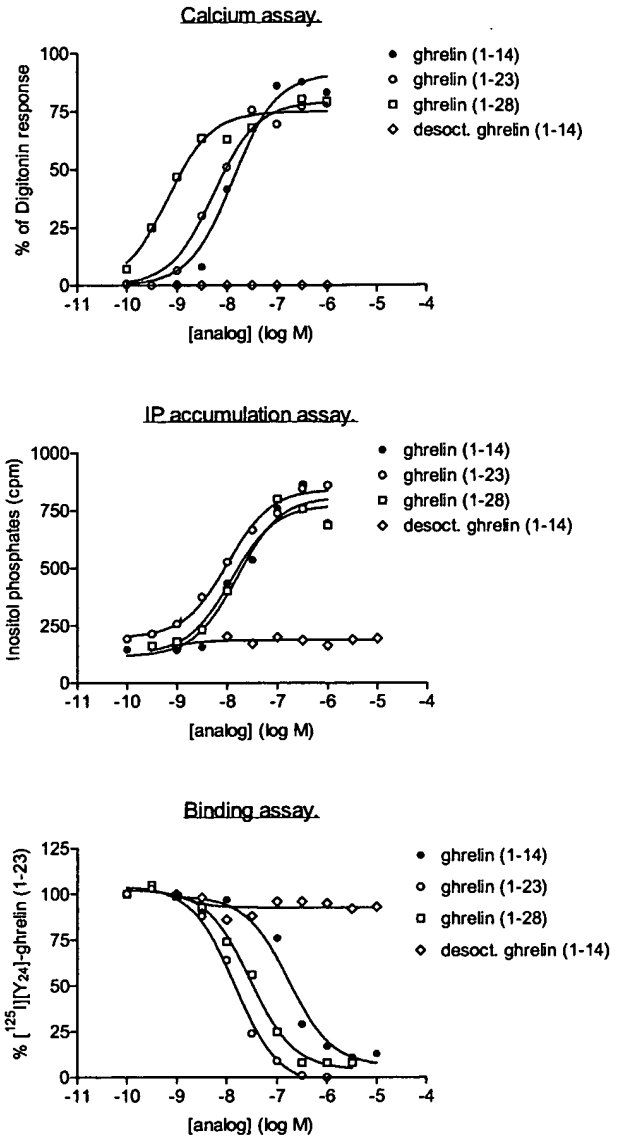


Fig. 1. Binding and functional properties of ghrelin (1–14) (●), ghrelin (1–23) (○), ghrelin (1–28) (□) and desoctanoylated ghrelin (1–14) (◇). Data are expressed as the percentage of the maximal response (Digitonin) for calcium assay (upper panel), as the quantity of inositol phosphates produced (cpm) for IP accumulation assay (middle panel), and as the percentage of specific $[^{125}I][Y_{24}]$-ghrelin (1–23) bound in the absence of a competing peptide for binding assay (lower panel). One typical experiment is represented of three.

inositol phosphates determinations, but in the latter case the $Ala^4$ derivative was shifted so far to the right that the $EC_{50}$ value could not be validly determined. We also observed that the substitution of the Phe residue in position 4 by Tyr, prepared in the course of the development of an iodinated tracer (see below), led to a markedly reduced potency (Table 1, Fig. 2) and $pIC_{50}$ (Table 1, Fig. 3), while the same substitution in positions 8, 14 and 24 had little effect or improved the peptide's affinity for the receptor.

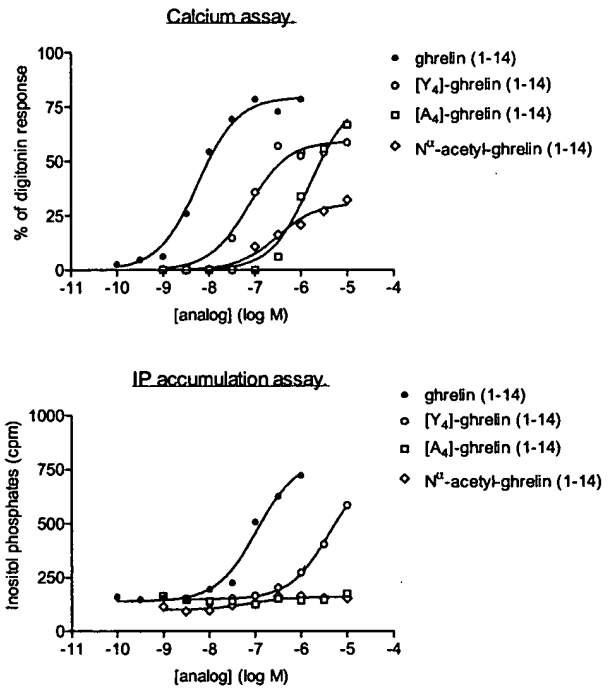


Fig. 2. Activation of the wild-type GHS-receptor by ghrelin (1–14) (●), [$Y_4$]-ghrelin (1–14) (○), [$A_4$]-ghrelin (1–14) (□), and $N^{\alpha}$-acetyl-ghrelin (1–14) (◇). Data are represented as the percentage of the maximal response (Digitonin) for the calcium assay (upper panel) and as the quantity of inositol phosphates produced (cpm) for the IP accumulation assay (lower panel). One typical experiment is represented of three.

### 3.3. Iodinated [$Y_{24}$]-ghrelin (1–23) tracer for receptor identification and competition binding curves

Ghrelin does not harbor a Tyr residue allowing easy iodination (G-S-[Octa]$S_3$-F-L-S-P-E-H-Q-R-V-Q-Q-R-K-E-S-K-K-P-P-A-K-L-Q-P-R), but iodination on the $His^9$ residue has been reported [12]. To obtain an appropriate tracer, we introduced a Tyr residue in selected positions (positions 4, 8, 14 and 24) and evaluated the unlabeled peptide analogs. [$Y_4$]-ghrelin (1–14) had a lower potency and affinity, whereas [$Y_8$]-ghrelin (1–14), [$Y_{14}$]-ghrelin (1–13) and [$Y_{24}$]-ghrelin (1–23) had high potencies ranging between 1 to 10 nM and were successfully iodinated. As mentioned above, the longer ghrelin forms (i.e. (1–23) and (1–28)) were significantly better than (1–14) ghrelin, and [$Y_{24}$]-ghrelin (1–23) compared to ghrelin (1–23) or ghrelin (1–28), was not significantly different. In the present work, we therefore used iodinated [$Y_{24}$]-ghrelin (1–23) for binding studies.

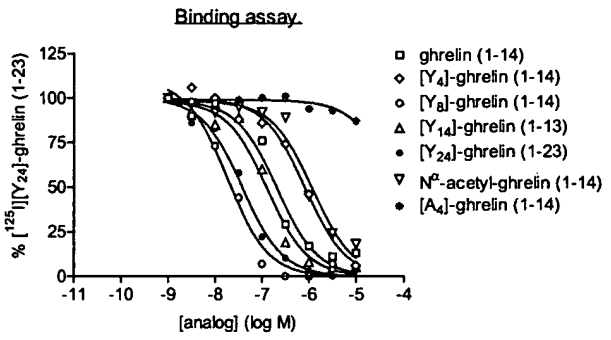


Fig. 3. Competition curves with [$^{125}$I][$Y_{24}$]-ghrelin (1–23) as tracer, by ghrelin (1–14) (□), [$Y_4$]-ghrelin (1–14) (◇), [$Y_8$]-ghrelin (1–14) (○), [$Y_{14}$]-ghrelin (1–13) (△), [$Y_{24}$]-ghrelin (1–23) (●), $N^{\alpha}$-acetyl-ghrelin (1–14) (▽) and [$A_4$]-ghrelin (1–14) (*) to the wild-type receptor, GHS-R. Data are expressed as the percentage of specific [$^{125}$I][$Y_{24}$]-ghrelin (1–23) bound in the absence of competitive peptide. One typical experiment is represented of three.

### 3.4. Evaluation of the non-ghrelin analog GHRP-6

GHRP-6 was tested as a representative of the short non-ghrelin agonists. GHRP-6 was the most potent stimulus for calcium mobilization, but was not significantly different from ghrelin (1–14) in binding and in IP assays (Table 1).

### 3.5. Importance of the free $NH_3^+$ on peptide activity

N-terminally acetylated ghrelin (1–14) ($N^{\alpha}$-acetyl-ghrelin (1–14)) was 20-fold less potent than the 1–14 peptide with

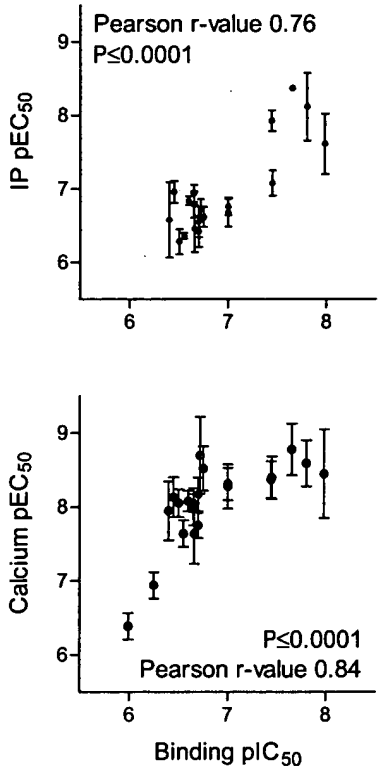


Fig. 4. Graphical correlation between IP $pEC_{50}$ and binding $pIC_{50}$ (upper panel) and intracellular calcium level $pEC_{50}$ and binding $pIC_{50}$ (lower panel). Correlation was determined using the Pearson test. Statistical test values are reported on the graphs.

respect to $[Ca^{2+}]_i$, and was the only modification presenting a significantly reduced maximal efficacy (40% of the response to ghrelin (1–14)). Inositol phosphates potency could not be determined due to its very low efficacy (Fig. 2). Binding data displayed a fivefold right shift.

Nevertheless, as shown in Fig. 4, for all peptides tested for which $pEC_{50}$ values could be determined, calcium mobilization and IP $pEC_{50}$ values were correlated to the binding $pIC_{50}$ values (Fig. 4) with a Pearson $r$ value of 0.76 and 0.84 ($P < 0.0001$), respectively.

## 4. Discussion

Studies of the GHS-receptor are based on different ligands: non-peptide compounds such as MK-0677, non-ghrelin peptides with D-amino acid residues such as GHRP-6 or hexarelin, and, more recently, ghrelin and fragments thereof [23]. Likewise, most studies address tissue samples rather than the cloned receptor.

Indeed, in cardiomyocytes and in endothelial cells, ghrelin and desoctanoylated ghrelin inhibited apoptosis by activation of extracellular signal-regulated kinase-1/2 and Akt serine kinases [2], yet the GHS-receptor was not detected in these cells. Furthermore, in competitive binding studies using the [$^{125}$I]-labeled Tyr-Ala-hexarelin on cardiomyocytes, the pharmacology of the binding site was different from that of the cloned GHS-receptor [18], as was the binding protein's molecular weight [5]. Even in the best characterized ghrelin target, i.e. the pituitary gland, identification of the GHS-receptor seemed difficult as [$^{125}$I]Tyr-Ala-hexarelin labeled, in the pituitary and hypothalamus, approximately 30-fold more sites than [$^{125}$I][$Y_4$]-ghrelin [16], leading the authors to suggest that only some hexarelin receptors are ghrelin receptors. However, short ghrelin analogs such as the octanoylated pentapeptides that are fully active on the recombinant GHS-receptor [3,11] were unable to displace [$^{125}$I][$Y_4$]-ghrelin [16] from pituitary membranes.

Thus, to determine the critical residues contained in the amino-terminal moiety of ghrelin, for functional signaling and receptor recognition we performed an Ala-scan between residues 1 and 14 and synthesized several other analogues. The stable expression of the cloned GHS-receptor as a CHO-aequorin cell line allowed us to measure intracellular calcium mobilization and IP accumulation. We could therefore address functional studies on model cell lines, and carry out binding experiments in response to analogs, outside any "constitutive" responses elicited by receptors distinct from the known recombinant ghrelin receptor under study.

We confirmed the essential role of octanoylation of Ser$^3$ for receptor recognition [14], and the function of Gly$^1$Ser$^2$ as a spacer between the α-amino and octanoylated-Ser$^3$ groups [15], as the [$A_1$]- and [$A_2$]-ghrelin analogues were not significantly different from ghrelin (1–14) in IP and binding studies. In contrast, the $N^{\alpha}$-acetyl-ghrelin (1–14) was the only low affinity analog with a partial agonist behavior, strongly suggesting that the positive charge at the amino-terminus was required for efficient binding and activation [15].

In addition, our data reveal that Phe$^4$ residue is crucial for receptor recognition. Indeed, the [$A_4$]-ghrelin and [$Y_4$]-ghrelin analogues yielded significantly reduced IP and calcium mobilization $pEC_{50}$ values ($P < 0.001$), supporting a direct interaction of Phe$^4$ with the receptor. The ghrelin analogs modified in position 4 were probably full agonists, as extrapolated from calcium stimulation curves. These results compellingly invalidate [$^{125}$I][$Y_4$]-ghrelin as an appropriate tracer for GHS-receptor binding studies. Therefore, we prepared other ghrelin derivatives with Tyr residues in positions 8, 14 and 24 that were at least as active as the corresponding ghrelin fragments.

Using the new tracer [$Y_{24}$]-ghrelin (1–23), with an affinity and a biological activity comparable to that of full-length ghrelin on the recombinant receptor, we obtained robust binding experiments in transiently transfected HEK cells. Consequently, iodinated [$Y_{24}$]-ghrelin (1–23) is an efficient ghrelin peptide tracer and a noteworthy alternative to the non-ghrelin $^{35}$S-labeled MK-0677 for ghrelin receptor labeling and certainly more suited than the [$Y_4$]-ghrelin tracers given the newly attributed importance of the fourth ghrelin residue.

Remarkably, our results clearly demonstrated that all positions between 1 and 14 of ghrelin—except for the α-amino group, octanoylated Ser$^3$ and Phe$^4$ side chains—could be modified with little or no effect on receptor recognition and activation. This corroborated the observation by Matsumoto et al. [15] that ghrelin (1–5) amide is almost as active as full length ghrelin. Furthermore, the NMR-derived structures of ghrelin and ghrelin (1–5) amide were very similar. Strangely enough, Glu$^8$ did not seem ideal for receptor recognition, as Ala$^8$ and Tyr$^8$ ghrelin (1–14) analogues were more potent than the ghrelin (1–14) reference fragment. Biological activity thus cannot account for the high degree of conservation of ghrelin amongst the mammalian peptide sequences determined to date, i.e. 90% identity within the first 20 N-terminal residues. We propose that an alternative explanation may be that the conservative pressure stems from the need to maintain a specific peptide context required for ghrelin to be recognized and serve as the proper substrate for the protein or protein complex performing the octanoylation reaction.

## Acknowledgments

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# Ghrelin and truncated ghrelin variant plasmid vectors administration into skeletal muscle augments long-term growth in rats

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**Abstract**

Ghrelin is an acylated peptide recently identified as an endogenous ligand for the growth hormone (GH) secretagogues (GHSs) receptor (GHS-R) and is involved in a novel system for regulating GH release. To study the biological activities of ghrelin using plasmid vector administration, we constructed myogenic expression vectors containing the full length cDNA of swine ghrelin-28 (pGEM-wt-sGhln) and truncated variant (pGEM-tmt-sGhln) consisting of the first seven residues of ghrelin (including $Ser^3$ substituted with $Trp^3$) with addition of a basic amino acid, Lys (K) at the C-terminus. After intramuscular injection of pGEM-wt-sGhln and pGEM-tmt-sGhln, RT-PCR analysis demonstrated that the ectopic expressions of ghrelin and its variant were observed 30 days post-injection. The level of GH increased in rat serum, and was significantly higher than that of the control group 20 days post-injection with pGEM-tmt-sGhln ($P < 0.05$). Administration of 150 μg of pGEM-wt-sGhln and pGEM-tmt-sGhln enhanced growth in rats over 30 days and great stimulatory responses were observed at day 10 and 20 post-injection respectively, whose body weight gains were on average 15% ($P < 0.05$) and 21% $P < 0.033$ significantly heavier than controls. These results suggested that skeletal muscle might have the potential to perform post-translational acylation for ghrelin, and short ghrelin variant might have the biological effects as wild type ghrelin.


*Keywords:* Ghrelin; Truncated ghrelin variant; GH; Myogenic vector

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## 1. Introduction

Growth hormone (GH) is an anabolic hormone that enhances protein synthesis, lypolysis, and epiphyseal growth, and is implicated in the regulation of the immune system. Regulated expression of the GH pathway is essential for optimal linear growth, as well as for homeostasis of carbohydrate, protein and fat metabolism [1]. The pulsatile release of GH from the pituitary somatotrophs is regulated at least by two hypothalamic neuropeptides: growth hormone-releasing hormone (GHRH) and somatostatin (SS) [2], and possibly by ghrelin [3].

Ghrelin, an endogenous ligand for growth hormone (GH) secretagogues (GHS) receptor (GHS-R), has been originally discovered predominantly in endocrine cells within the rat stomach. This 28-amino acid peptide possesses a unique serine residue at the third position ($Ser^3$) that is modified by *n*-octanoic acid. Acylation is essential for ligand binding to the receptor and subsequent ghrelin activities [3]. Recently, it has also been reported that short peptide encompassing the first 4 and 5 residues (including acylated serine) of ghrelin is the " active core" required for its efficient receptor binding and activation [4,5]. Ghrelin has been shown to exert a very potent and specific GH-releasing activity both in vitro and in vivo [6,7] and function in energy metabolism [8–10].

Interestingly, calculated at equal mass dose or in molar concentration, ghrelin appears to be much more potent than GHRH in stimulating GH secretion [11]. Current studies on that GH-releasing activity of synthetic ghrelin [8,12] or GHS [13] in vivo require the frequently subcutaneous, intravenous or intracerebroventricular infusion at higher dose for the short half-life of the peptide in vivo. However, it is possible that, like GHRH, extracranially secreted ghrelin, as a mature or truncated form maybe is biologically active and stimulates GH secretion at a low level [14,15]. These characteristics would make ghrelin an excellent candidate for gene therapy.

Direct plasmid DNA transfer is currently the basis of many emerging therapeutic strategies because it avoids the potential problems with vial vector or lipid particles. Skeletal muscle is a preferred target tissue because the skeletal muscle fibril has long life span and can be transduced by circular DNA plasmid [16]. Skeletal muscle borne plasmids have been demonstrated to express efficiently over months [17]. In this study, we describe the characterization of growth-promoting myogenic expression constructs (pGEM-wt-sGhln and pGEM-tmt-sGhln) in vivo. Intramuscular injections of pGEM-wt-sGhln and pGEM-tmt-sGhln enhanced rat growth greatly, and increased GH serum level in rats significantly 20 days post-injection with pGEM-tmt-sGhln as compared with control animals.

## 2. Materials and methods

### *2.1. Cloning of porcine cDNA for ghrelin and truncated ghrelin variant*

The two overlapping oligonucleotides, 100 bp (sense) and 96 bp (antisense) respectively were synthesized (Sangon, Shanghai, China) according to porcine preproghrelin mRNA sequence (GeneBank accession no. AF308930). A stop codon and a SpeI site were incorporated into the 3′ end of antisense oligo (5′-GCAACTAGTCTA-3′). In the porcine ghrelin-28 matured peptide, the codon for the fifth amino acid, Leu (L), TTG was mutated

with TTA to create the AflII site. Two oligos were annealed in a total volume 20 μl reaction with 2.5 μM of each oligo, 1 mM of dNTP, 0.25 U/μl of Taq DNA polymerase by 94 °C, 4 min, 58 °C, 40 s, and 72 °C, 1 min, in the first cycle, and subsequently amplified with a primer pair (sense: 5′-ATGCCCTCCACGGGGACCATTT-3′ and antisense: 5′-GCAACTAGTCTACCGGGGCTTC-3′). The PCR product was cloned to sequencing vector pMD18-T (Takara) to yield pMD18-wt-sGhln containing cDNA of signal peptide and ghrelin-28. For the mutant variant, the PCR reaction was performed using pMD18-wt-sGhln as the template and a primer pair (sense: 5′-TTCGAGCTCGGTACCCGG-3′ and antisense: 5′-GGGGCTTAAGAACCAGGAGC-3′). The product of PCR was inserted into SacI/AflII sites of pMD18-wt-sGhln to create pMD18-mt-sGhln containing mutant cDNA of ghrelin in which the $Ser^3$ was substituted with $Trp^3$. For the truncated variant, the pMD18-T-mt-sGhln was double cut by SacI/AflII, and small fragments were recovered, and then amplified from this small fragment with a primer pair (sense: 5′- TTCGAGCTCGGTACCCGG-3′ and antisense: 5′- GCAACTAGTCTACTTGGGGCTTAA-3′). The product of PCR was inserted into SacI/SpeI sites of pMD18-mt-sGhln to yield pMD18-tmt-sGhln which contains the cDNA for the truncated porcine ghrelin variant consisting the first seven residues of ghrelin (including $Ser^3$ substituted with $Trp^3$) and an additional basic amino acid, Lys at the C-terminus. The conditions of PCR all above were the same in 30 cycles with initial denatured step at 94 °C for 5 min, 30 s annealing at 60 °C, 30 s, elongation at 72 °C and 30 s denaturing at 94 °C.

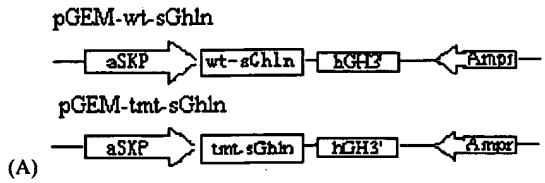
### 2.2. Construction of myogenic expression vectors and purification

The plasmid DNA backbone pGEM-A5f3f was reformed from pGEM-5zf (Promega). The pGEM-A5f3f contains a 2857 bp NcoI/MluI fragment, which includes 1910 bp porcine skeletal muscle α-actin (SKA) 5′-flanking region, the first exon and the first intron [18], and the 3′ untranslated region of hGH cDNA in a 613 bp SacI/NsiI fragment. To construct the eukaryotic expression vector, pGEM-A5f3f was blunt ended the MluI site after digestion using Klenow large fragment (Takara) and digested with SpeI. This vector was used to ligate with the fragment with two compatible sites either from the wild-type ghrelin or the truncated ghrelin variant cDNAs to create pGEM-wt-sGhln and pGEM-tmt-sGhln. The two recombinant constructs were ascertained by sequence analysis. The schematic maps of pGEM-wt-sGhln and pGEM-tmt-sGhln and the amino acid sequence of ghrelin, short ghrelin analog encoded by the constructs were shown in Fig. 1.

Plasmids were extracted and purified with Q sepharose XL in ÄKTA platform (Amersham Bioscience) and dissolved in water to final concentration of 1–2 mg/ml. The purity and concentration of the plasmids were confirmed by 1% of agarose gel electrophoresis and measured by UV absorption at 260 and 280 nm. Levels of endotoxin in the preparation were less than 100 endotoxin units (EU/mg plasmid). The percentage of supercoiled DNA and OD260/280 ratio of these preparations were in the range of >95–98% and 1.8–1.9, respectively.

### 2.3. Intramuscular injection of expression vectors in rats

Sprague–Dawley (SD) male SPF rats (Beijing Vital Laboratory Animal Technology Company Ltd., PR China) were housed and cared for in the animal facility of Institute for Space




(A)

pGEM-wt-sGhln

MPSTGTICSLLLLSVLLMADLAMA*GSSFLSPEHQKVQQRKESKKPAAKLKPR

pGEM-tmt-sGhln

(B) MPSTGTICSLLLLSVLLMADLAMA*GSWFLSPK

Fig. 1. The schematic maps of pGEM-wt-sGhln and pGEM-tmt-sGhln constructs and the deduced amino acid sequence of swine ghrelin and short ghrelin variant encoded by the constructs. (A) All constructs contain porcine skeletal α-actin promoter (aSKP) and the 3' untranslated region (UTR) of hGH. (B) Peptide structure of swine ghrelin and truncated variant encoded by corresponding expression vectors. The truncated porcine ghrelin variant encompasses the first seven residues of ghrelin (including $Ser^3$ substituted with $Trp^3$) with addition a basic amino acid, Lys (K) in the C-terminus. Amino acids before the symbol (*) represent signal peptide of ghrelin.

Medical Engineering (Beijing, China). All animal experimentations were performed in accordance with guidelines of institutional medical or animal research committees for the care and use of laboratory animals, under environmental condition of 12 h light/12 h darkness. On day −5, the left quadriceps muscle of rat (35–40 g of body weight) was injected with 100 μl of 0.75% bupivacaine [19,20] hydrochloride in saline solution. On day 0, the animals were weighed, and regenerating muscle was injected with 150 μg of pGEM-wt-sGhln, pGEM-tmt-sGhln or pGEM-A5f3f as a control in 150 μl phosphate buffer saline (PBS, pH 7.4), respectively. The animals were weighed and killed 5–30 days later. Blood samples were collected via transcardic after the animal was anesthetized, centrifuged after overnight at 4 °C, and stored at −80 °C prior to analysis. The treated and control muscles were removed and frozen in liquid nitrogen.

### *2.4. Gene expression analysis by RT-PCR*

Total RNAs from the injected muscles were extracted using TRIzol reagent (Invitrogen Inc., CA). Total RNA was treated by DNaseI and 1 μg of total RNA was used in the reverse transcriptase reaction, using M-MLVRT (Life Technology Inc.) according to manufacturer's instruction. In negative control (−) reaction, the reverse transcriptase was not included. Specific oligonucleotides were used to amplify either 168bp fragment of pGEM-wt-sGhln cDNA with sense primer: 5′-ATGCCCTCCACGGGGACCATTT-3′ and antisense primer: 5′-GCAACTAGTCTACCGGGGCTTC-3′ or 96 bp fragment of pGEM-tmt-sGhln cDNA with a sense primer: 5′-ATGCCCTCCACGGGGACCATTT-3′ and antisense primer: 5′-GGGCTTAAGAACCAGGA-3′. The RT-PCR conditions were 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s in 25 μl volume. A 630 bp fragment of rat β-actin cDNA as control was performed as described before [21].

### 2.5. Rat GH RIA

Rat serum GH (rGH) concentrations were measured by radioimmunoassay (RIA) using kit provided by the NHPP, NIDDK, NICHHD, USDA, USA. Values are expressed in terms of rat GH-RP-2 standard (potency 2 IU/mg) as ng/ml of serum. Blood sample taken every 15 min over 2 h period 10, 20 and 30 days following the initial plasmid injections were assayed and a mean 2 h GH concentration was calculated. The sensitivity of the assay was 0.1 ng/ml. Intra assay variability was 6%. To avoid inter assay variations, all samples from each experiment were run in one single assay.

### 2.6. Statistical analysis

Data were analyzed with the General Linear Models Procedure of SAS software. Values are expressed as mean ± standard error of the mean (S.E.M.). The statistical significance of differences between plasmid DNA injection groups and control group was evaluated independently with Ducan's *t*-test for multiple comparisons, preceded by the analysis of variance (ANOVA). A *P*-value of less than 0.05 was considered to be significant.

## 3. Results

### 3.1. Tissue expression of pGEM-wt-sGhln and pGEM-tmt-sGhln post-injection

The expressions of pGEM-wt-sGhln and pGEM-tmt-sGhln in vivo were assayed by RT-PCR with RNA extracted from injected muscles (Fig. 2). Total RNA was treated by DNase I to eliminate any contamination from the injected plasmid and 1 μg of total RNA was used in the reverse transcriptase reaction. RT-PCR analysis showed that the in vivo

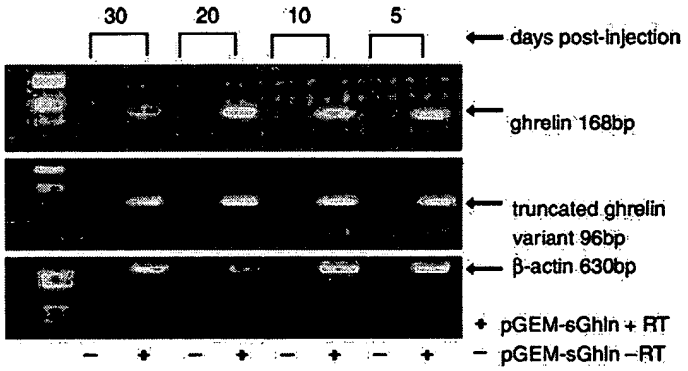



Fig. 2. Expression of ghrelin and truncated ghrelin variant by RT-PCR analysis after injection of 150 μg pGEM-wt-sGhln and pGEM-tmt-sGhln into left quadriceps muscle of adult rats. Upper panel: PCR reaction using pGEM-wt-sGhln cDNA specific oligonucleotides; middle panel: PCR reaction using pGEM-tmt-sGhln cDNA specific oligonucleotides; lower panel: PCR reaction using rat cytoskeletal β-actin cDNA specific oligonucleotides. In (−) tubes the reverse transcriptase was not included.

expression of the pGEM-wt-sGhln (168 bp PCR fragment) and the pGEM-tmt-sGhln (96 bp PCR fragment) was detectable from day 5–30 post-injection when amplified with specific primer pairs. A 630 bp PCR fragment of rat cytoskeleton β-actin was used as a control. The efficiency of DNase treatment to eliminate plasmid DNA was determined by using RNA from tissue detected when the reverse transcriptase was absent from the reaction and no amplification was observed.

### *3.2. GH-releasing activity in rats*

In order to assess the effects of expression of pGEM-wt-sGhln and pGEM-tmt-sGhln, the levels of serum rGH in the injected animals were evaluated to compare with the PBS-injected control group (Fig. 3). Blood sample taken every 15 min over 2 h period 10, 20 and 30 days following the initial plasmid injections were assayed and a mean 2 h GH concentration was calculated. Time course analysis of rGH showed that stimulations were day 10 post-injection (pGEM-wt-sGhln, 57 ± 6.7 ng/ml; pGEM-tmt-sGhln, 52 ± 7.4 ng/ml; control, 45 ± 7.2 ng/ml of $n = 10$), and greater stimulations were 20 days after injection (pGEM-wt-sGhln, 59 ± 10.8 ng/ml; pGEM-tmt-sGhln, 73 ± 11.3 ng/ml; controls, 37 ± 10.5 ng/ml; $n = 10$,). The serum rGH day 20 post-injection was statistically significant for pGEM-tmt-sGhln injected rats compared with controls ($P < 0.043$). By day 30 post-injection, serum rGH returned gradually to baseline (pGEM-wt-sGhln, 10 ± 2.6; pGEM-tmt-sGhln, 9 ± 2.07 ng/ml; controls, 9 ± 2.0 ng/ml; $n = 10$) respectively.

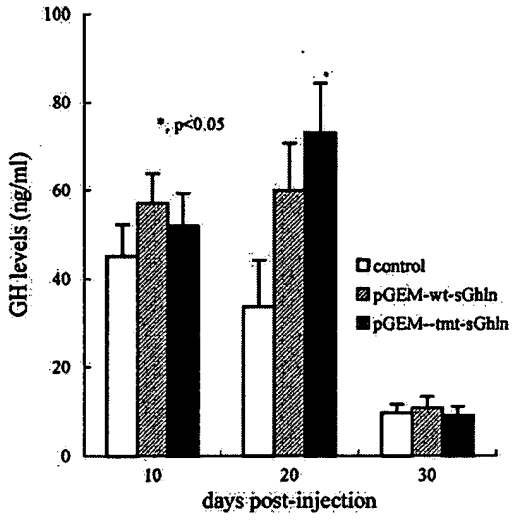



Fig. 3. Serum rGH concentration in rats measured by RIA after direct intramuscular injection of pGEM-wt-sGhln ($n = 10$), pGEM-tmt-sGhln ($n = 10$) and age-matched PBS-injected control animals ($n = 10$). The results are presented as means ± S.E.M. The serum rGH concentrations of pGEM-tmt-sGhln injected animals were significantly higher than PBS-injected control groups 20 days post-injection (* $P = 0.05$).

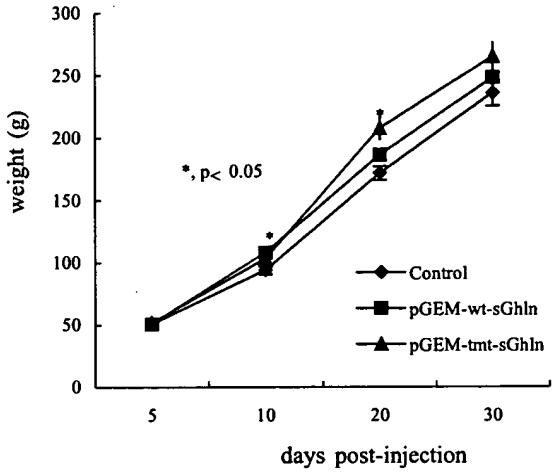



Fig. 4. Increase in body weight after a single dose plasmid DNA injection in the regenerating left quadriceps muscle of rats. The results are presented as means ± S.E.M. Injection of pGEM-wt-sGhln ($n = 10$), pGEM-tmt-sGhln ($n = 10$), and age-matched PBS-injected control animals ($n = 10$). $^*P = 0.05$ was observed at day 10 post-injection of pGEM-wt-sGhln and at day 20 post-injection of pGEM-tmt-sGhln compared with PBS-injected control group.

### 3.3. *Promotion of growth by myogenic expression vectors*

Since the endogenous GH has been stimulated by the injection of these constructs, growth rate of such animals could be also affected or promoted. To assess this issue, weights of these animals were measured over 30 days. The growth rate from group injected with pGEM-wt-sGhln or pGEM-tmt-sGhln was augmented over 30 days (Fig. 4) and increases were appreciated by day 10 and 20 post-injection, respectively. The total average gains of body weight from the groups pGEM-wt-sGhln and pGEM-tmt-sGhln injections were 15 and 21% heavier over the controls ($108 \pm 2.9$ g versus $93 \pm 3.2$ g, $P < 0.05$; $207 \pm 9.0$ g versus $171 \pm 5.6$ g, $P < 0.033$). These results are consistent with the expression of constructs as well as the released GH activities in serum and suggested that the wild type of ghrelin gene in expressing construct can effectively augment the biological growth of animal by ex vivo inoculation. More importantly, significant animal growth is exhibited by ex vivo expression of the truncated variant of ghrelin gene construct and suggests that the first seven amino acids and a modification represent the function region of ghrelin gene.

## 4. Discussion

In this study, we have demonstrated the feasibility of delivering a peripheral hormone, swine ghrelin or truncated ghrelin variant, via muscle-specific expression vectors. Injection of pGEM-wt-sGhln encoding mature porcine ghrelin augments rat growth suggesting that skeletal muscle may have the ability to posttranslationally acylate the ectopically expressed

ghrelin. Injection of pGEM-tmt-sGhln encoding the truncated serine$^3$ residue substitution ghrelin variant increases the growth greatly in rats. It suggests that the truncated ghrelin variant has the biological activities as that of wild type ghrelin. This might be due to that the truncated and key amino acid substitution (Ser$^3$ replaced with Trp$^3$) ghrelin has a higher efficiency for the receptor binding and subsequent activation, though there still exist in controversies whether the C-terminal part of the ghrelin molecule plays a role in establishing the bioactive conformation of the intact acylated ghrelin molecule [22]. In addition, low immunogenecity of this form in vivo may also be a factor. Furthermore, insertion of a basic amino acid, Lys (K) in the C-terminus of truncated ghrelin variant may also increase the biological activities in vivo as in vitro [5]. However, these speculations above will be needed to further confirm. The sequences before the seventh amino acid of ghrelin are conserved in all the species examined so far, and our results are in agreement with the finding that N-terminal is also the active core required for ghrelin using plasmid vector, thus the truncated ghrelin molecule, which displays a high degree of GH secretory activity in rats, may also be useful in farm animals and even in human clinical medicine. Linear growth velocity and body composition respond to GH replacement therapies in a broad spectrum of conditions, both in humans and in farm animals. In domestic livestock, GH stimulate milk production, increase feed-to-milk conversion, and sustain growth, primarily by increasing lean body mass, and increase overall feed efficiency. Thus ghrelin and truncated ghrelin variant plasmid administration may provide an alternative to classical recombinant GH proteins treatment. It will be also interest to find out whether co-injection of the ghrelin or the ghrelin truncated variant in combination with GHRH gene or their fusion genes are more efficient in augmenting the growth [23–25], and could greatly reduce the quantity of ghrelin plasmid DNA needed to achieve the physiological levels of GH.

Transgenic expression in skeletal muscle has been demonstrated using plasmid backbone and reporter genes driven by porcine skeletal SKA promoters [26]. One potential limitation for the SKA promoter to direct gene transfer is that, in general, it yields a lower level of expression in vivo than that of a viral promoter derived from CMV. In order to achieve favorable physiological changes such as weight gain and improved body composition at low dose of plasmid used in a large mammal, we believed that it would be necessary to increase the efficiency of therapeutic plasmid DNA delivery system such as utilizing a high efficient muscle-specific promoter [27,28] or by optimizing the electroporation method [29]. The control of cellular access and uptake, intracellular trafficking and nuclear retention of plasmid must also be achieved to improve efficiency of plasmid-based gene transfer [30].

In addition, treated rats did not experience any adverse effects of therapy, had normal biological profiles and developed without any notable side-effect [15,29]. The intramuscular plasmid delivery may be efficacious in improving performance of domestic animals and constitute a step forward to human gene delivery.

## Acknowledgements

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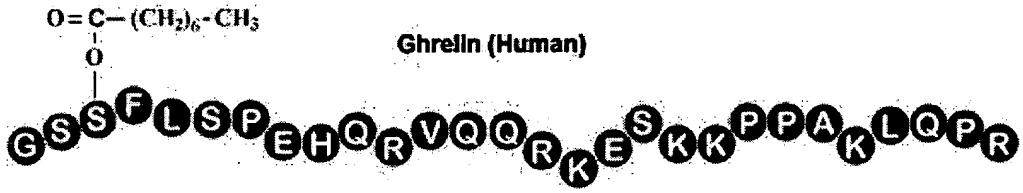
## Scientists Report Active Ghrelin Fragments and Ghrelin Antagonist

*Ghrelin: Endogenous Growth-Hormone Releasing Peptide with Novel Regulatory Mechanism*

Human growth hormone (hGH) is secreted throughout life. hGH is implicated as an important factor in the aging process due to its significant decrease over the life cycle, and its involvement in many processes, such as fat, protein, carbohydrate, muscle, and bone metabolism. Therefore, hGH has been hailed by the popular press as the fountain of youth.




Small synthetic molecules called growth-hormone secretagogues (GHSs) stimulate the release of growth hormone (GH) from the pituitary through human secretagogue receptor 1a (hGHSR1a) (1, 2, 3). The endogenous ligand for this receptor was identified in 1999 as **ghrelin** (4). Kojima and co-workers reported **ghrelin** to be an octanoylated, 28-residue peptide with the n-octanoyl group at $Ser^3$, the first observed to date. **Ghrelin** stimulates GH-release from rat primary cultured pituitary cells in a dose-dependent manner ($EC_{50}$ = 2.1 nM) and it induces an increase of intracellular $Ca^{2+}$ in GHS-R-expressing cells with $EC_{50}$ of 2.5 nM (4). Rat and human peptide sequences are identical except for the amino acid substitutions at positions 11 and 12.

Although **ghrelin** and the known hypothalamic peptide, growth-hormone releasing factor, stimulate GH-release, they differ both in GH secretion mechanism and in a structural aspect, the octanoyl group attached on the side chain of $Ser^3$ in ghrelin is essential for expressing activity. This octanoylated posttranslational modification is the first of this type observed to date. The major ghrelin-producing tissue is the stomach and ghrelin immunoreactivity is found in healthy human blood. Recently Merck scientists, Bednarek and coworkers, report that only the first 5 residues are necessary to maintain the activity of endogenous **ghrelin**, but a large hydrophobic group on the $Ser^3$ side chain is still required for activity (3).

This peptide may constitute a new regulatory mechanism for GH-release. It is conceivable that **ghrelin** may have other functions in some tissues other than pituitary, because the GHS receptor is expressed in heart, lung, pancreas, intestine, and adipose tissue.

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### New! Ghrelin Antagonist and Non-Acylated Ghrelin Now Available

Recently, Holst and coworkers reported a substance P analog to be a low potency ghrelin antagonist. In 1988, **[D-Arg$^1$,D-Phe$^5$,D-Trp$^{7,9}$,Leu$^{11}$]-Substance P** was originally reported by Woll *et al.* to be a potent bombesin antagonist able to inhibit small cell lung cancer growth *in vitro*.

Now the Danish researchers have found this ghrelin antagonist to be a full inverse agonist with an $EC_{50}$ = 5.2 nM.

Also, Broglio and coworkers recently reported des-octanoyl ghrelin peptide did not possess endocrine activity in animal models. However, **Des-*n*-Octanoyl-[Ser$^3$]-Ghrelin (Human)** was shown to be as effective as ghrelin in exhibiting antiproliferative effects on tumor cell lines in *in vitro* studies.

These new products, non-acylated ghrelin and ghrelin antagonist, should serve as a valuable research tool to aid diabetes and obesity research efforts. In addition, ghrelin active and inactive (negative control) fragments, are available from Peptides International

| CODE | ***New Ghrelin Products*** **Ghrelin PDF Brochure** | QTY | USD |
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| PGH-3656-PI **NEW!** | **H-His-D-Trp-D-Lys-Trp-D-Phe-Lys-$NH_2$**<br>[D-Lys$^3$]-Growth Hormone Releasing Peptide-6 (GHRP-6)<br>(M.W. 930.12) $C_{49}H_{63}N_{13}O_6$<br>*Ghrelin Antagonist*<br>*L. Pinilla, M.L. Barreiro, M. Tena-Sempere, and E. Aguilar, Neuroendocrinology, 77, 83 (2003).* | 1 mg<br>5 mg | 25<br>49 |
| PGH-3653-PI **NEW!** | **Des-*n*-Octanoyl-[Ser$^3$]-Ghrelin (Human)**<br>Non-Acylated Ghrelin (Human)<br>Gly-Ser-Ser-Phe-Leu-Ser-Pro-Glu-His-Gln-Arg-Val-Gln-Gln-Arg-Lys-Glu-Ser-Lys-Lys-Pro-Pro-Ala-Lys-Leu-Gln-Pro-Arg<br>(M.W. 3244.74) $C_{141}H_{235}N_{47}O_{41}$<br>*Inactive Ghrelin*<br>F. Broglio, A. Benso, C Gottero, F. Prodam, C. Gauna, L. Filtri, E. Arvat, A.J. van der Lely, R. Deghengi, and E. Ghigo, *J. Endocrin. Invest.*, **26**, 192 (2003). | 0.5 mg | 145 |
| PGH-3654-PI **NEW!** | **Des-*n*-Octanoyl-[Ser$^3$]-Ghrelin (Rat)**<br>Non-Acylated Ghrelin (Rat)<br>Gly-Ser-Ser-Phe-Leu-Ser-Pro-Glu-His-Gln-Lys-Ala-Gln-Gln-Arg-Lys-Glu-Ser-Lys-Lys-Pro-Pro-Ala-Lys-Leu-Gln-Pro-Arg | 0.5 mg | 145 |

(M.W. 3188.67) $C_{139}H_{231}N_{45}O_{41}$
*Inactive Ghrelin*

| CODE | Active Fragments of Ghrelin | QTY | USD |
|---|---|---|---|
| PGH-3625-PI | **Ghrelin (Human, 1-18)**<br>Gly-Ser-Ser(*n*-Octanoyl)-Phe-Leu-Ser-Pro-Glu-His-Gln-Arg-Val-Gln-Gln-Arg-Lys-Glu-Ser-$NH_2$ | 1 mg<br>5 mg | 95<br>285 |
| PGH-3626-PI | **Ghrelin (Human, 1-14)**<br>Gly-Ser-Ser(*n*-Octanoyl)-Phe-Leu-Ser-Pro-Glu-His-Gln-Arg-Val-Gln-Gln-OH | 1 mg<br>5 mg | 75<br>225 |
| PGH-3627-PI | **Ghrelin (Human, Rat, 1-10)**<br>Gly-Ser-Ser(*n*-Octanoyl)-Phe-Leu-Ser-Pro-Glu-His-Gln-$NH_2$ | 1 mg<br>5 mg | 60<br>180 |
| PGH-3628-PI | **Ghrelin (Human, Rat, 1-5)**<br>Gly-Ser-Ser(*n*-Octanoyl)-Phe-Leu-$NH_2$ | 1 mg<br>5 mg | 45<br>135 |

## *Negative Control Ghrelin Fragments*

**Inactive Ghrelin without n-Octanoyl on Serine[3]**

| | | | |
|---|---|---|---|
| PGH-3645-PI<br>**NEW!** | **Des-*n*-Octanoyl-[Ser[3]]-Ghrelin (Human, 1-18)**<br>H-Gly-Ser-Ser-Phe-Leu-Ser-Pro-Glu-His-Gln-Arg-Val-Gln-Gln-Arg-Lys-Glu-Ser-$NH_2$<br>*Negative Control for Ghrelin (Human, 1-18)* | 1 mg<br>5 mg | 65<br>195 |
| PGH-3646-PI<br>**NEW!** | **Des-*n*-Octanoyl-[Ser[3]]-Ghrelin (Human, 1-14)**<br>H-Gly-Ser-Ser-Phe-Leu-Ser-Pro-Glu-His-Gln-Arg-Val-Gln-Gln-OH<br>*Negative Control for Ghrelin (Human, 1-14)* | 1 mg<br>5 mg | 55<br>155 |
| PGH-3647-PI<br>**NEW!** | **Des-*n*-Octanoyl-[Ser[3]]-Ghrelin (Human, Rat, 1-10)**<br>H-Gly-Ser-Ser-Phe-Leu-Ser-Pro-Glu-His-Gln-$NH_2$<br>*Negative Control for Ghrelin (Human, 1-10)* | 1 mg<br>5 mg | 45<br>125 |
| PGH-3648-PI<br>**NEW!** | **Des-*n*-Octanoyl-[Ser[3]]-Ghrelin (Human, Rat, 1-5)**<br>H-Gly-Ser-Ser-Phe-Leu-$NH_2$<br>*Negative Control for Ghrelin (Human, 1-5)* | 1 mg<br>5 mg | 35<br>95 |

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| | | | |
|---|---|---|---|
| PGH-4372-s | **Ghrelin (Human)**<br>Gly-Ser-Ser(n-Octanoyl)-Phe-Leu-Ser-Pro-Glu-His-Gln-<br>Arg-Val-Gln-Gln-Arg-Lys-Glu-Ser-Lys-Lys-Pro-Pro-Ala-<br>Lys-Leu-Gln-Pro-Arg<br>(M.W. 3370.9) $C_{149}H_{249}N_{47}O_{42}$<br>*Endogenous Growth-Hormone Releasing Peptide with Novel Regulatory Mechanism* | 0.1 mg vial | 235 |

1) M. Kojima, H. Hosoda, Y. Date, M. Nakazato, H. Matsuo, and K. Kangawa,
*Nature*, **402**, 656 (1999). *(Original)*
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| | | | |
|---|---|---|---|
| PGH-4373-s | **Ghrelin (Rat)**<br>Gly-Ser-Ser(n-Octanoyl)-Phe-Leu-Ser-Pro-Glu-His-Gln-<br>Lys-Ala-Gln-Gln-Arg-Lys-Glu-Ser-Lys-Lys-Pro-Pro-Ala-<br>Lys-Leu-Gln-Pro-Arg<br>(M.W. 3314.8) $C_{147}H_{245}N_{45}O_{42}$<br>*Endogenous Growth-Hormone Releasing Peptide with Novel Regulatory Mechanism*<br>1) M. Kojima, H. Hosoda, Y. Date, M. Nakazato, H. Matsuo, and K. Kangawa,<br>*Nature*, **402**, 656 (1999). *(Original)*<br>•This compound is distributed through the Peptide Institute under license agreement with Dr. Kangawa. | 0.1 mg vial | 235 |
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| B-031-68 | Ghrelin (1-18) Amide (Human) (O-n-Octanoyl-Ser3) - Biotin Labeled | 10 µg | 300 | Order |
| 031-75 | Ghrelin (1-18) Free Acid (Human) (O-n-Octanoyl-Ser3) | 100 µg | 200 | Order |
| 031-74 | Ghrelin (1-23) Amide (Human) (O-n-Octanoyl-Ser3) | 100 µg | 200 | Order |
| 031-76 | Ghrelin (1-23) Free Acid (Human) (O-n-Octanoyl-Ser3) | 100 µg | 200 | Order |
| 031-81 | Ghrelin (1-27), (O-n-Decanoyl-Ser3) (Human) | 100 ug | 180 | Order |
| B-031-81 | Ghrelin (1-27), (O-n-Decanoyl-Ser3) (Human) - Biotin Labeled | 10 µg | 300 | Order |
| 031-80 | Ghrelin (1-27), (O-n-Octanoyl-Ser3) (Human) | 100 ug | 180 | Order |
| B-031-80 | Ghrelin (1-27), (O-n-Octanoyl-Ser3) (Human) - Biotin Labeled | 10 ug | 300 | Order |
| 031-83 | Ghrelin (1-27), Ser3 (Human) | 100 ug | 180 | Order |
| B-031-83 | Ghrelin (1-27), Ser3 (Human) - Biotin Labeled | 10 ug | 300 | Order |
| 031-67 | Ghrelin (1-4) Amide (O-n-Octanoyl-Ser3) | 200 µg | 100 | Order |
| 031-42 | Ghrelin (1-5) Amide (Des-Octanoyl-Ser3) (Human, Rat) | 200 µg | 30 | Order |
| 031-41 | Ghrelin (1-5) Amide (Human, Rat) | 200 µg | 60 | Order |
| 031-72 | Ghrelin (1-7) Amide (Human) (O-n-Octanoyl-Ser3) | 100 µg | 120 | Order |
| | Ghrelin (1-7) Amide (Human) (O-n-Octanoyl-Ser3) - Biotin Labeled | | 250 | |

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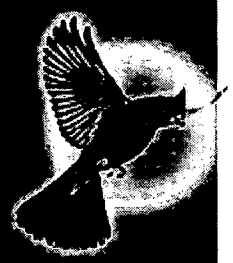
|---|---|---|---|---|
| B-031-72 | | 10 µg | | Order |
| 031-66 | Ghrelin (1-7)-Lys Amide (O-n-Octanoyl-Ser3) | 200 µg | 100 | Order |
| B-031-66 | Ghrelin (1-7)-Lys Amide (O-n-Octanoyl-Ser3) - Biotin Labeled | 10 µg | 300 | Order |
| 031-70 | Ghrelin (1-9) Amide (Human) (O-n-Octanoyl-Ser3) | 100 µg | 150 | Order |
| 031-65 | Ghrelin (1-9), Gly8 (O-n-Octanoyl-Ser3) | 200 µg | 100 | Order |
| 031-44 | Ghrelin (17-28)(Human, Rat) | 200 µg | 120 | Order |
| 031-61 | Ghrelin-21-C10 (Eel) (O-n-Decanoyl-Ser3) | 100 µg | 200 | Order |
| B-031-61 | Ghrelin-21-C10 (Eel) (O-n-Decanoyl-Ser3) - Biotin Labeled | 10 µg | 300 | Order |
| 031-60 | Ghrelin-21 (Eel) (O-n-Octanoyl-Ser3) | 100 µg | 180 | Order |
| 031-64 | Ghrelin (24-58), Gln51, Prepro (Human) | 200 µg | 220 | Order |
| B-031-64 | Ghrelin (24-58), Gln51, Prepro (Human) - Biotin Labeled | 10 µg | 350 | Order |
| 031-38 | Ghrelin (3-28)(Rat, Mouse) | 100 µg | 180 | Order |
| **031-77** | **Ghrelin (52-75), Prepro, (Human)** | 100 µg | 150 | Order |
| 031-79 | Ghrelin (52-75),Tyr0, Prepro, (Human) | 100 µg | 150 | Order |
| 031-78 | Ghrelin (52-76), Cys76, Prepro(Human) | 100 µg | 150 | Order |
| 031-35 | Ghrelin (52-85) -Prepro (Human) | 100 µg | 140 | Order |
| 031-37 | Ghrelin (52-85) -Prepro (Rat, Mouse) | 100 µg | 140 | Order |
| 031-36 | Ghrelin (86-117) -Prepro (Rat, Mouse) | 100 µg | 140 | Order |
| 031-69 | Ghrelin Amide (Tilapia) (O-n-Decanoyl-Ser3) | 100 µg | 200 | Order |
| B-031-69 | Ghrelin Amide (Tilapia) (O-n-Decanoyl-Ser3) - Biotin Labeled | 10 µg | 300 | Order |
| 031-43 | Ghrelin C-terminal Hexapeptide | 200 µg | 50 | Order |
| **031-50** | **Ghrelin (Canine)** | 100 µg | 180 | Order |
| 031-58 | Ghrelin [Dap-Octanoyl 3](Human) | 100 µg | 180 | Order |
| 031-51 | Ghrelin [Des-Gln14](Canine) | 100 µg | 180 | Order |
| 031-46 | Ghrelin [Des-Gln14](Des-Octanoyl3)(Rat, Mouse) | 100 µg | 120 | Order |
| 031-47 | Ghrelin [Des-Octanoyl](1-18) Motilin-Related Peptide (Human) | 100 µg | 140 | Order |
| 031-48 | Ghrelin [Des-Octanoyl](1-18) Motilin-Related Peptide (Rat, Mouse) | 100 µg | 140 | Order |
| 031-33 | Ghrelin [Des-Octanoyl3](Rat, Mouse) | 100 µg | 140 | |



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| B-031-33 | Ghrelin [Des-Octanoyl3](Rat, Mouse), Biotin Labeled | Please CALL | Call for Price | Order |
| 031-45 | Ghrelin [Des-Q14-](Rat, Mouse) | 100 µg | 180 | Order |
| B-031-63 | Ghrelin, Gln28 (Human), Biotin Labeled | 20 µg | 300 | Order |
| 031-63 | Ghrelin, Gln28 (Human) (O-n-Octanoyl-Ser3) | 100 µg | 180 | Order |
| **031-30** | **Ghrelin (Human)** | 100 µg | 95 | Order |
| 031-57 | Ghrelin [Lys29 –[Epsilon]-Biotinyl, Ser3(Des-Octanoyl)] (Human) | 100 µg | 250 | Order |
| 031-56 | Ghrelin [Lys29](Human) | 100ug | 250 | Order |
| 031-32 | Ghrelin [Non-Acylated] / Ghrelin [Des-Octanoyl-Ser3] (Human) | 100 µg | 120 | Order |
| 031-82 | Ghrelin, (O-n-Decanoyl-Ser3) (Human) | 100 ug | 180 | Order |
| B-031-82 | Ghrelin, (O-n-Decanoyl-Ser3) (Human) - Biotin Labeled | 10 ug | 300 | Order |
| **031-52** | **Ghrelin (Porcine)** | 100 µg | 180 | Order |
| **031-34** | **Ghrelin -Prepro (86-117)(Human)** | 100 µg | 140 | Order |
| 031-77 | Ghrelin, Prepro(52-75) (Human) | 100ug | 150 | Order |
| **031-31** | **Ghrelin (Rat, Mouse)** | 100 µg | 95 | Order |
| 031-55 | Ghrelin [Tyr29] [Des-Octanoyl](Human) | 100 µg | 200 | Order |
| 031-54 | Ghrelin [Tyr29](Human) | 100 µg | 250 | Order |

Found **64** products for **Ghrelin**. Showing from **1** to **64** in alphabetical order.